Future developments

Future developments: *In vitro* growth (IVG) of human ovarian follicles

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Disclosure:
Evelyn Telfer has no conflicts of interest to declare. Work relating to this area in the Telfer laboratory has been funded by the Medical Research Council.

Key words: Oocyte/Follicle Culture/Primordial Follicles/IVG/IVA/IVM/

Key Message: In Vitro Growth (IVG) of immature oocytes from cryo-preserved ovarian cortex has the potential to produce mature oocytes but is still at an experimental stage. Further research is required before clinical application can be realised

Abstract

Removal and storage of ovarian cortical tissue is currently offered to young female cancer patients undergoing potentially sterilizing chemotherapy and/or radiotherapy. For patients at high risk of re-introduction of malignancy through auto-transplantation, the ultimate aim is to achieve complete oocyte development from this tissue *in vitro*. The ability to develop human oocytes from the earliest follicular stages through to maturation and fertilisation *in vitro* would revolutionise fertility preservation practice. This has been achieved in mouse where *in vitro* grown (IVG) oocytes from primordial follicles have resulted in the production of live offspring. Systems that support growth and development of oocytes from human ovarian cortex are being developed by several groups. This review focuses on the steps required to recapitulate *in vitro* the process of human oocyte development from the primordial stage and the systems currently available to support this.

Introduction

The ability to develop human immature oocytes *in vitro* would have many potential applications but would be of particular relevance to Fertility Preservation. Ovarian tissue cryopreservation is now an option for women with cancer prior to undergoing gonadotoxic treatments (1). Re-implantation of cryo-preserved tissue is currently the only option to use stored tissue but in many cases, particularly for women with Leukemia, malignant cells are present in the ovary therefore, re-implantation is not an option, however, the oocytes within this tissue could potentially be grown *in vitro* (1). Culture systems with the aim of achieving *in vitro* growth (IVG) of immature oocytes to maturity and subsequent fertilization *in vitro* (IVF) have been the subject of research for almost 40 years. Several systems that support the growth of later stages of follicle development from rodents have been developed (2-8) with some reporting the production of live young (3, 4, 6-8). Complete development from the most immature oocytes (primordial stages) *in vitro* with subsequent IVF of oocytes followed by embryo transfer and production of offspring has been achieved in the mouse using a two step culture system (9, 10). The initial studies resulted in the birth of one mouse which developed abnormalities as an adult (9). Following alterations to the culture medium several mouse embryos and offspring have been obtained using IVG oocytes then combined with *in vitro* maturation (IVM) and IVF (10). More recently, *in vitro* systems that support complete development of murine oocytes starting from induced pluripotent stem cells (11) and from primordial germ cells (12) have been reported and these systems have resulted in competent oocytes that produce embryos and live young.

The work on rodents has provided proof of principle and has encouraged the challenge of adapting these systems to support human oocyte development *in vitro*. IVG of primordial follicles would be of particular benefit to pre-pubertal girls undergoing fertility preservation before being exposed to damaging chemotherapy (13). Currently the only fertility preservation option for pre-pubertal girls is storage of ovarian cortical tissue with the potential for re-implantation at a later time (13). In cases where re-implantation is not possible, IVG could provide another option to restore fertility. For IVG to be clinically viable, the process would need to start with primordial follicles as cortical strips that are stored contain predominantly this stage. Whilst there still remains much detailed research to be carried out before IVG of primordial follicles could ever be clinically applied, a great deal of progress has been made in developing culture techniques to support human oocyte

development *in vitro*. In this review the current status of human IVG from primordial follicles will be considered.

Stages of Follicle Development

The majority of follicles within the ovary in all young mammalian females will be at the primordial stage of development and these follicles are continually utilised throughout reproductive life (14). It is not known whether the pool of primordial follicles represents a homogeneous population but at this stage follicles have not yet been exposed to selection processes that lead to follicle degeneration (15, 16). Primordial follicles represent a "resting" population of germ cells (oocyte arrested at dictyate stage of Prophase 1 of meiosis surrounded by a few flattened granulosa cells), that are formed pre-natally. Recruitment into growth takes place throughout the woman's reproductive life and a sequence of precisely regulated processes is required for complete oocyte/follicle development to occur. The sequence starts with (a) initiation of primordial follicle growth and development to the preantral follicle stage; (b) growth of the preantral stage with formation of a fluid filled cavity (antrum) and expansion to the pre ovulatory or Graafian follicle stage (c) rupture of the Graafian follicle releasing a cumulus-oocyte complex at ovulation in response to the mid-cycle LH surge (17, 18).

The oocyte is held in meiotic arrest as it grows within the follicle and it must acquire the ability to resume meiosis (meiotic competence) as well as the ability to support fertilisation and embryonic development (developmental competence). Oocyte development is dependent upon the environment of the individual follicle for its function as a gamete and this is regulated by inhibitory and stimulatory endocrine, paracrine and autocrine signalling by the somatic cells of the follicle (granulosa and surrounding theca cells) enhanced by several oocyte specific factors mediated through bi-directional communication (19, 20). The physiological requirements of the oocyte, granulosa and theca cells are extremely complex and dynamic therefore recapitulating the process of follicle activation and growth *in vitro* is one of the greatest technical challenges in reproductive technology (18).

Growing Human follicles *in vitro*

Several approaches have been taken to support early human follicle development *in vitro* starting with primordial follicle activation (21-31). Whilst there are several culture systems that support a specific stage of human oocyte development *in vitro*, there is so far only one

report that supports human primordial follicles to the stage of meiotic maturation (30). To achieve complete development of human oocytes *in vitro* a multi-step culture system is required (26, 30, 32). The first step in this process is to facilitate the initiation of primordial follicle development and support early growth; the second step is to optimise the growth of follicles from preantral to antral stages; step 3 supports the completion of oocyte growth ready for *in vitro* maturation in step 4 (Figure 1). In optimising a culture system to obtain developmentally competent oocytes the focus should be on oocyte development and this may preclude the need to develop large follicular structures *in vitro*. The multi-step approach needs to support the changing requirements of the developing oocyte and its surrounding somatic (granulosa) cells whilst maintaining good oocyte-somatic cell interactions. Therefore, providing conditions that support the maintenance of appropriately differentiated somatic cells in contact with the developing oocyte similar to the oocyte granulosa cell complexes in the rodent system is essential (4, 10).

IVA: *In vitro* Activation of human primordial follicles

The majority of follicles within ovarian cortical tissue will be at the quiescent primordial stage. Activation of primordial follicles *in vitro* (IVA) and early follicle development are key features of any IVG system. Human primordial follicles can be activated and grow well within mechanically loosened cortical pieces, developing to multilaminar preantral (secondary) stages within 6 days (26, 30). Central to this process is preparation of the ovarian tissue. This involves removal of most of the underlying stromal tissue and any growing follicles so that the cultured tissue consists of ovarian cortex containing primordial and primary follicles. When these small fragments of human ovarian cortex are cultured there is a significant shift of follicles from the quiescent to the growing pool over short periods of 6 – 10 days (26, 29-31).

It remains unclear how follicle activation is controlled but it is known to involve a combination of inhibitory, stimulatory and maintenance factors (33). The importance of the phosphatidylinositol-3'-kinase (PI3K-AKT) signalling pathway within the oocyte has been implicated in regulating activation of primordial follicles using mouse knockout models (34) and in human using culture of ovarian cortex (29, 35, 36). The phosphatase and tensin homolog deleted on chromosome ten (PTEN) acts as a negative regulator of this pathway and suppresses initiation of follicle development (34). The transcription factor Forkhead Protein O3 (FOXO3) is a downstream effector of this pathway and acts to inhibit follicle recruitment

(37). Other components of this pathway are dependent on the mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine kinase that regulates cell growth and proliferation in response to growth factors and nutrients and also regulates primordial follicle activation (38). From knockout mouse data it appears that whilst PTEN within the oocyte suppresses activation of primordial follicles mTORC promotes it. How these pathways regulate human follicle development is unclear but culture models facilitate the study of these processes (29, 36, 39).

Significant primordial follicle activation occurs in step one of the multi-step culture system (26, 30). This activation appears to be as a result of disrupting the Hippo signalling pathway during the preparation of the tissue (36, 40, 41). Hippo disruption increases expression of downstream growth factors but manipulation of the PI3K pathway results in further activation (29, 35, 36, 40, 41). Inhibition of PTEN in cultured human ovarian cortex results in increased activation of primordial follicles and more secondary follicles but subsequent growth and survival of isolated secondary follicles is compromised (29, 36). The detrimental effect on secondary follicles may be as a result of alterations in DNA damage and repair responses as demonstrated recently in a bovine culture model (42).

Cortical strip culture removes follicles from the *in vivo* endocrine and paracrine processes regulating growth rate; however, follicles will still be subject to the effect of follicle interactions and the influence of stromal cell factors. It is clear that tissue shape and stromal density are important factors that contribute to the regulation of follicle growth initiation in-vitro, as solid cubes of cortical tissue show lesser growth initiation (21) than cortex cultured as flattened "sheets," where much of the underlying stroma is removed (26, 30). The physical environment of the follicles within the cortical tissue affects their response to stimulatory and inhibitory factors and therefore influences their ability to grow (43). Once follicle growth has been initiated within cortical tissue they can develop to multi-laminar stages but do not survive well within the cortical environment as growth is inhibited resulting in loss of follicle integrity and oocyte survival (22, 26). Growing follicles need to be released from the cortical stromal environment and cultured individually to limit the effect of follicle interactions (26, 30, 31, 44).

IVG: *In vitro* Growth of human preantral follicles

Preantral follicles can be isolated from cortical tissue post culture by mechanical dissection, enzymatic isolation or a combination of both. Collagenase and DNase can be used to remove preantral follicles from stromal tissue, however collagenase can cause damage which leads to poor survival of follicles (45). The presence of theca layers is required for growing follicles to retain their structure and survive the second stage of IVG and these may be compromised by collagenase (45). More purified enzyme preparations such as Liberase may avoid the damage that occurs with Collagenase (46, 47). Mechanical isolation of follicles has the advantage of preserving follicular integrity by maintaining the basal lamina and thecal layers, however the procedure is laborious and results in a low yield (26, 30).

Supporting the growth of human preantral follicles *in vitro* has led to the development of matrices to maintain follicle structure. Alginate hydrogels has been used to encapsulate human preantral follicles and support their growth *in vitro* (48). Alginate encapsulation is thought to mimic the extra cellular matrix *in vivo* in terms of its ability to facilitate molecular exchange between the follicle and the culture medium whilst its flexibility can accommodate cell proliferation but its rigidity prevents dissociation of the follicular unit. The rigidity of the alginate capsule affects follicle development as inhibition of growth and reduced steroidogenesis have been reported in murine follicles embedded in 1% alginate gels (49) whereas fully grown human oocytes have been produced using 0.5% gels (48).

The application of tissue engineering to support the growth of isolated follicles has been making progress; decellularized ovarian tissue and 3D micro-porous scaffolds are being explored as matrices to support preantral follicle growth (50, 51). Recent work has explored the production of electrospun patterned porous scaffolds which may be more accessible and reproducible than decellularised tissue (52). Engineered scaffolds clearly have great potential and should be developed further to support human follicle growth *in vitro*.

The multi-step culture system that has been developed for human follicles (30) does not use matrices or scaffolds to support the growth of isolated preantral follicles. Isolated growing follicles are cultured in v-shaped micro-well plates and this has supported follicular architecture *in vitro* whilst promoting growth, differentiation and antral formation (26, 30).

Once follicles are isolated from the ovarian cortex their progression *in vitro* is remarkable. Secondary human follicles isolated enzymatically from fresh ovarian tissue and cultured in the presence of Follicle Stimulating Hormone (FSH), become steroidogenically active and

complete oocyte growth within 30 days (48) and these oocytes have been shown to be capable of meiotic maturation (53). Primordial follicles grown within fragments of ovarian cortex to multi-laminar stages which are then isolated without the use of enzymes and cultured in the presence of Activin and FSH become steroidogenic within 10 days of *in vitro* growth (26, 30).

During step 2 of the multi-step system, isolated follicles cultured individually form antral cavities within 6-8 days. At this stage oocyte-granulosa cell complexes can be retrieved by applying gentle pressure to the follicle. Complexes with complete cumulus and adherent mural granulosa cells are selected for step 3 of the multi-step system (Figure 1) (30). Step 3 involves culturing the complexes on membranes in the presence of Activin-A and rhFSH for up to 4 days until oocytes reach a diameter of 100 microns (30).

That *in vitro* grown follicles can produce fully grown oocytes after a relatively short culture period confirms that local ovarian factors inhibit follicle development *in vivo*. Oocyte size is an indicator of its ability to resume meiosis therefore sustaining oocyte growth is the major objective of any complete *in vitro* development system (17). There are clearly differences in growth rate depending on whether the whole follicle is cultured (48, 53) or whether, complexes are removed for further growth after an antral cavity has formed (26, 30). Whether the growth rate observed *in vitro* should be characterised as accelerated is not clear. The rate observed represents uninhibited growth without brakes that are required *in vivo* to regulate follicle development within the context of the reproductive cycle. Comparisons of culture systems is needed to determine optimal conditions but at this time there is only one complete system that supports growth from human primordial to maturation (30). The next step is to determine whether the growth pattern *in vitro* can support the development of healthy oocytes or whether it is deleterious to oocyte function, epigenetic changes and health.

Meiotic Maturation of Oocytes from IVG human follicles

The final stage in the IVG process before IVF can take place is IVM to support resumption of meiosis to the point of Metaphase II (Figure 1). IVM has been a successful strategy for embryo production in domestic species and has been applied to human oocytes with varying degrees of success (54, 55). The first live birth after IVM of immature oocytes was reported in 1991 (56), although IVM was being utilised during the early development of IVF (57). IVM is performed in a limited number of centres and success rates vary with the main factor

being oocyte source and stage (55). The rate of maturation of immature oocytes remains below that of oocytes harvested from stimulated ovaries, indicating that the protocols are sub-optimal or many of the harvested oocytes are intrinsically unable to undergo maturation (54, 55).

IVG oocytes derived from the multistep culture system undergo meiotic maturation following an IVM protocol (30). These oocytes form Metaphase II spindles but emit abnormally large polar bodies (30). Polar body size is influenced by the proximity of the spindle to the oocyte cortex and the inter-chromosomal spacing within Metaphase II spindles (58). If there is a loss of spindle contact with the oocyte cortex this can lead to extrusion of large polar bodies (59). The cause of the large polar bodies in the IVG derived oocytes is not known but it indicates that culture conditions need to be further optimised. The consequences of such abnormalities on chromosome balance in mature oocytes needs to be investigated.

Whilst acknowledging that there are no fully optimised culture systems for human oocytes there is now proof of concept that complete *in vitro* growth of human oocytes is possible (30). The end point of any IVG system is to produce developmentally competent and epigenetically normal oocytes therefore future research needs to focus on optimising each of the stages and to gain further understanding of the epigenetic status of IVG oocytes and of any embryos formed (60).

Summary

The most significant clinical application of *in vitro* growth (IVG) of human oocytes is in Fertility Preservation given the widespread adoption of ovarian tissue cryopreservation for cancer patients (1). It is clear that making a good egg is not an easy or straightforward process (18). If reliable methodology could produce *in vitro* generated mature human oocytes capable of fertilisation this would be a viable alternative to autologous transplantation. Apart from the clinical implications and potential of the various *in vitro* growth systems; each of them provide access to the process of human oogenesis in an experimentally tractable paradigm. Through these systems we will gain greater understanding of human oocyte development which will ultimately lead to improvements in Fertility Preservation.

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List of Abbreviations:

FSH	Follicle Stimulating Hormone
FOXO3	Forkhead box protein O3
IVA	In Vitro Activation
IVG	In Vitro Growth
IVF	In Vitro Fertilisation
IVM	In Vitro Maturation
PI3K-AKT	Phosphatidylinositol-3'-kinase and Protein Kinase B
PTEN	The phosphatase and tensin homolog deleted on chromosome ten
mTORC1	Mammalian target of rapamycin complex 1

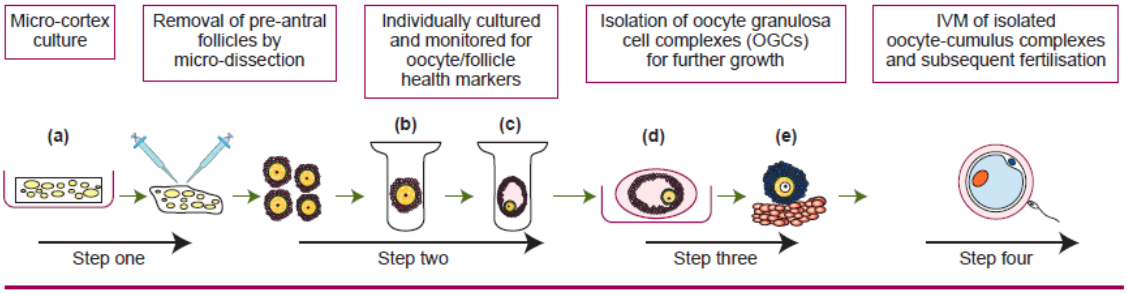

Figure 1: Diagrammatic representation of a multi-step culture system to support in vitro growth (IVG) of oocytes from human primordial follicles through to maturation as described by McLaughlin et al., 2018. Step 1, in vitro activation within micro-cortex for 7 days (a) then micro-dissection of multi-laminar growing follicles to be placed in step 2 (b) and cultured individually until antral formation occurs (c) Step 3, Isolation of the oocyte granulosa complex (d) from the intact follicle for further growth on membranes for up to 4 days (e). Step 4, Oocyte-Cumulus complexes placed within medium for in vitro maturation (IVM). Oocytes are then analysed for the presence of a Metaphase II spindle and a polar body. Fertilisation of IVG human oocytes has not yet been tested.